¹³C tracer analysis identifies extensive recycling of endogenous CO₂ in vivo

Likun Duan¹, Daniel E. Cooper², Grace Scheidemantle¹, Jason W. Locasale³, David G. Kirsch^{2,3}, and Xiaojing Liu^{1,*}

¹Department of Molecular and Structure Biochemistry, NC State University, Raleigh, NC 27695 USA

²Department of Radiation Oncology, Duke University School of Medicine, Durham, NC 27708 USA

³Department of Pharmacology and Cancer Biology, Duke University, Durham, NC 27708 USA

*Corresponding author: Xiaojing Liu, xliu68@ncsu.edu

Running title: Endogenous CO₂ recycling

Keywords: ¹³C tracing, high resolution mass spectrometry, anaplerotic metabolism, pyruvate carboxylase

Abstract

^{13}C tracing analysis is increasingly used to monitor cellular metabolism in vivo and in intact cells, but data interpretation is still the key element to unveil the complexity of metabolic activities. We have performed $[\text{U-}^{13}\text{C}]$ -glucose and $[\text{U-}^{13}\text{C}]$ -glutamine tracing in sarcoma-bearing mice (in vivo) and in cancer cell lines (in vitro). ^{13}C enrichment of metabolites in cultured cells and tissues was determined by liquid chromatography coupled with high-resolution mass spectrometer (LC-HRMS). As expected, citrate M+2 or M+4 is the dominant mass isotopologue in vitro. However, citrate M+1 was unexpectedly the dominant isotopologue in mice receiving $[\text{U-}^{13}\text{C}]$ -glucose or $[\text{U-}^{13}\text{C}]$ -glutamine infusion. One plausible explanation is that $^{13}\text{CO}_2$ produced from the oxidation of ^{13}C tracers in vitro is negligible due to the dilution of HCO_3^- supplemented to cell culture when sodium bicarbonate is used and diffusible volume of CO_2 in the culture incubator, while endogenous $^{13}\text{CO}_2$ in vivo is substantial and is fixed into the TCA cycle, purine, and serine, resulting in M+1 isotopologues. A time course study shows the generation of high abundance citrate M+1 early in plasma, which may serve as a potent non-invasive biomarker of tissue pyruvate carboxylase activity. Altogether, our results show that recycling of endogenous CO_2 is substantial in vivo and provides important insights into the experimental design and data interpretation of ^{13}C tracing assays.

Introduction

Cellular metabolites are in dynamic homeostasis. Metabolomics is an emerging tool to measure metabolic changes that represent the dynamic status of the cell, tissue, or whole organism and facilitate a better understanding of biological processes from a global level ¹. However, metabolomics mainly measures metabolite concentrations or the changes of metabolite levels, which reflect the net outcome of various metabolic pathways in which each metabolite is involved in, and it does not provide information on individual pathways that contribute to the production and disappearance of a specific metabolite. In contrast, stable isotope tracing provides information on metabolite dynamics and generates data on specific metabolic pathways. Stable isotope tracing has been used to probe specific metabolic pathways in various biological systems since the last century, soon after the initial isolation of isotopic tracers ². Over the past decades, advances in instrumentation and data analysis software enabled fast data acquisition and determination of isotope labeling patterns ¹. However, the data from isotope tracing experiments do not provide easily interpretable results, and interpreting ¹³C metabolite labeling patterns remains the limiting step of using stable isotope tracing to address biological questions ³. To extract maximal information from ¹³C tracing experiments and draw accurate conclusions, it is important to understand which labeling patterns represent different metabolic activities. Understanding the ¹³C transformations can help identify unexpected metabolic pathways of biological significance. For example, [U-¹³C]-glutamine would produce citrate M+4 following standard glutamine metabolism pathways, which involve glutaminase-mediated conversion to glutamate and then α -ketoglutarate, followed by oxidative metabolism after entering the TCA cycle

⁴. The observation of citrate M+5 suggests the existence of an alternative glutamine metabolism route, the reductive carboxylation of α -ketoglutarate, the reverse flux relative to the canonical oxidative TCA flux ⁵⁻⁶. This pathway has been demonstrated to be important for tumor growth under hypoxic conditions or tumors with mitochondrial defects and may serve as a potential therapeutic target ⁵⁻⁶. One challenge of interpreting ¹³C labeling patterns comes from the effects of culturing conditions or tissue microenvironment on cell or tissue metabolism. For instance, factors such as nutrient availability, oxygen level, or tissue context-dependent microenvironment, can greatly impact cellular metabolism ⁷ and consequently isotopic labeling of metabolites. Cells or isolated tissues fed with ¹³C tracers ex vivo also tend to have different labeling patterns from the same type of tissues of animals receiving in vivo tracing ^{1, 8}. These factors result in difficulties in data interpretation and developing new approaches to decipher labeling patterns.

Pyruvate carboxylase, a mitochondrial enzyme that catalyzes the production of oxaloacetate by combining pyruvate and bicarbonate, is one of the major anaplerotic enzymes. Pyruvate carboxylase supports gluconeogenesis in the liver and kidney by providing oxaloacetate. Pyruvate carboxylase is important for de novo synthesis of fatty acid in adipocyte tissues and is also involved in de novo synthesis of a neurotransmitter, glutamate, in astrocytes. Studies have shown that pyruvate carboxylation is required for tumor growth when glutamine metabolism is suppressed ⁹⁻¹⁰. ¹³C tracing has been used to study TCA anaplerosis in vitro and in vivo and citrate M+3 has been used to monitor pyruvate carboxylase activity after [U-¹³C]-glucose tracing ¹⁰⁻¹¹. For instance, under cell

culture conditions, pyruvate M+3 can enter the TCA cycle via pyruvate carboxylase and lead to the appearance of citrate M+3. Indeed, citrate M+3 is observed in cultured cells fed with [U-¹³C]-glucose. Nevertheless, citrate M+1 has been reported to be more abundant than M+3 in lung cancer, liver, and kidney after continuous infusion of [U-¹³C]-glucose or dietary delivery of [U-¹³C]-glucose in vivo^{8, 11}, but it remains poorly understood why M+1 is more abundant in vivo.

Here we report the distinct ¹³C labeling patterns of TCA intermediates in cultured cells, in-vivo tumors, and non-tumor tissues after [U-¹³C]-glucose or [U-¹³C]-glutamine tracing. We identified M+1-labeling of TCA intermediates are the most abundant species in vivo, but not in vitro. This finding is consistent with the results of in vivo [U-¹³C]-glucose tracing performed by other investigators^{8, 11-13}. We hypothesized that endogenous CO₂ is labeled by ¹³C tracers and subsequently used to produce M+1 isotopologues of TCA metabolites. To test this hypothesis, we traced the product of other CO₂-consuming reactions such as purine biosynthesis and measured adenosine M+1 in tumor and non-tumor tissues. We then compared the time course of M+1 and M+3 TCA intermediates and observed that M+1 citrate appeared earlier than M+3, which further suggests that CO₂ is produced endogenously and fixed into the TCA cycle. These findings provide a new paradigm to understand carbon atom transformations in vivo and should be taken into account when developing mathematic models to better reflect carbon flux.

Results

M+1-labeling of TCA metabolites is dominant in the in vivo tumor but not in in vitro cultured cells fed with ^{13}C glutamine tracer

To investigate the ^{13}C labeling patterns of TCA intermediates in cancer cells (in vitro), and in animals (in vivo) with [U- ^{13}C]-glutamine, two different types of tracing experiments were performed (Fig.1A). Sarcoma cells, generated from primary mouse sarcomas, were incubated in RPMI 1640 containing 2 mM [U- ^{13}C]-glutamine and 10% dialyzed FBS. Intracellular metabolites were extracted after 3 hours of tracing and subsequently analyzed using LC coupled with a high resolution mass spectrometer (LC-HRMS). Sarcoma-bearing mice were infused with [U- ^{13}C]-glutamine for 3 hours. At the end of infusions, sarcoma samples were collected and analyzed using LC-HRMS. ^{13}C enrichment was calculated based on metabolite peak area. Natural abundance correction was performed using software R with Bioconductor R package IsocorrectionR (21). In tumor (in vivo), [U- ^{13}C]-glutamine was converted into [$^{13}\text{C}_5$]-2-oxoglutarate (M+5) via glutaminolysis (Fig. 1B), followed by oxidative decarboxylation of 2-oxoglutarate M+5, resulting in succinate-CoA and succinate M+4. The M+4 carbon chain remains labeled in the next two reactions to produce malate M+4. Malate M+4 will be oxidized to oxaloacetate M+4, which is then used to generate citrate M+4. The citrate M+4 was then dehydrated by aconitase to give M+4 cis-aconitate. Isocitrate dehydrogenase (IDH) catalyzes the reversible reaction of isocitrate to alpha-ketoglutarate and CO_2 , and the reverse reaction contributes to the generation of citrate M+5 and cis-aconitate M+5 (Fig. 1B). In the cultured sarcoma cells, M+4 and M+5 isotopologues are the major species as expected. Surprisingly, unlike the labeling patterns in cultured cells, M+1-labeled intermediates are the major isotopologues in

mouse sarcomas in vivo. A plausible explanation is that decarboxylation of [U- ^{13}C]-glutamine produces $^{13}\text{CO}_2$, which is subsequently fixed into the TCA cycle in vivo. This model challenges the common assumption that endogenous $^{13}\text{CO}_2$ from decarboxylation of [U- ^{13}C]-glutamine is diluted by the body pool of bicarbonate and thus the contribution of $^{13}\text{CO}_2$ to ^{13}C -labeling is not significant. Furthermore, TCA reactions are often referred to as having a net flux in the clockwise direction. The observation of M+1-labeled succinate, fumarate, and malate indicates the reverse reactions and exchange fluxes from oxaloacetate to succinate.

M+1-labeled TCA metabolites by [U- ^{13}C]-glucose is dominant in the in-vivo tumor but not in cultured cells

To investigate whether glucose-driven CO_2 can also be recycled, we next performed in vitro and in vivo tracing experiments using [U- ^{13}C]-glucose (Fig. 2A). Through glycolysis, [U- ^{13}C]-glucose is converted into [$^{13}\text{C}_3$]-pyruvate (M+3), which then feeds a series of reactions in the TCA cycle. Pyruvate M+3 is first converted into acetyl-CoA, and the M+2 acetyl group of acetyl-CoA is then transferred to the four-carbon oxaloacetate to form citrate M+2 (Fig. 2B). The citrate M+2 is then dehydrated by aconitase to give cis-aconitate M+2, which subsequently goes through a series of chemical transformations to yield α -ketoglutarate M+2, losing one carboxyl group as CO_2 . After oxidative decarboxylation of α -ketoglutarate M+2, a four-carbon chain is generated as succinyl-CoA, which is further converted into succinate M+2. The second carbon lost as CO_2 is originated from the carbon of oxaloacetate. The succinate M+2 remains labeled in the next two enzymatic reactions to provide malate M+2. During the second round of the

TCA cycle, oxaloacetate M+2 reacts with acetyl-CoA M+2 and produces citrate M+4. Indeed, M+4 was detected in cultured sarcoma cells (in vitro) and in sarcomas in mice (in vivo) and M+4 is less abundant than M+2. Surprisingly, M+1 species are the dominant isotopologues for all the TCA metabolites in the tumor but not in the cultured cancer cells. Taken together, the appearance of high abundant M+1 TCA intermediates in [U-¹³C]-glutamine (Fig. 1) and [U-¹³C]-glucose tracing (Fig. 2) experiments can be explained by the recycling of endogenous ¹³CO₂.

M+1-labeled TCA intermediates is also observed in non-tumor tissues in vivo.

To investigate whether CO₂ recycling is a tumor-specific metabolic event or not, we further looked into the labeling patterns of TCA intermediates in non-tumor tissues. Similar to what we observed in tumor, citrate M+1, succinate M+1, and malate M+1 are dominant isotopologues in mouse liver and skeletal muscle (Fig. 3A). In fact, a similar result of TCA intermediate enrichment in normal tissues after infusing mice with [U-¹³C]-glucose was reported by other independent investigators¹³, but the source of the M+1 species was not identified. We downloaded the relevant source data from the supplementary material of this paper and analyzed the labeling pattern of citrate, succinate, and malate in different normal tissues (Fig. 3B). For citrate, the enrichment fractions of M+1 and M+2 are comparable. For succinate and malate, M+1 is even more abundant than M+2. These results indicate that endogenously generated ¹³CO₂ participates in anaplerotic metabolism in non-tumor tissues as well.

Endogenous CO₂ contributes to purine biosynthesis in vivo.

To explore whether CO₂ recycling participates in other metabolic events, we then looked into de novo purine synthesis, which consumes CO₂ (Fig. 4A). An intermediate in purine biosynthesis, aminoimidazole ribonucleotide (AIR) is combined with ¹³CO₂ by AIR carboxylase to produce CAIR M+1. The CAIR was then converted into (S)-2-[5-Amino-1-(5-phospho-D-ribosyl)imidazole-4-carboxamido]succinate (SAICAR) by SAICAR synthetase, followed by several concerted steps to yield inosinic acid M+1. The inosinic acid M+1 is then converted by 5'-ribonucleotide phosphohydrolase into inosine M+1, which is subsequently converted into adenosine M+1 by adenosine aminohydrolase. ¹³C labeling patterns of adenosine from sarcoma cells fed with [U-¹³C]-glutamine/[U-¹³C]-glucose in vitro and from sarcomas of sarcoma-bearing mice receiving [U-¹³C]-glutamine/[U-¹³C]-glucose infusion in vivo for 3 hours are shown in Fig. 4B. No substantial labeling of adenosine with [U-¹³C]-glutamine was observed in cultured cells. However, adenosine M+1 was detected in sarcomas from sarcoma-bearing mice receiving the [U-¹³C]-glutamine infusion, strongly suggesting that ¹³CO₂ produced from [U-¹³C]-glutamine participates in purine biosynthesis. The [U-¹³C]-glucose tracing leads to M+5 labeled-adenosine in both cultured cells and tumor, indicating the successful labeling of ribose 5-phosphate (R5P) by [U-¹³C]-glucose through the pentose phosphate pathway in vitro and in vivo. However, M+1-labeling of adenosine with [U-¹³C]-glucose was only observed in the tumor in vivo. The observation of adenosine M+1 in sarcomas after [U-¹³C]-glutamine or [U-¹³C]-glucose infusion in mice but not in cultured sarcoma cells is consistent with our previous observations that ¹³CO₂ recycling is substantial in vivo but not in vitro (Figs. 1-2).

Endogenous CO₂ contributes to serine biosynthesis in vivo.

We further investigated other ¹³C labeling patterns which can be explained by ¹³CO₂ recycling. In the [U-¹³C]-glutamine tracing experiment, serine M+1 was observed in mouse sarcomas in vivo but not in sarcoma cells cultured in a CO₂ incubator (Fig. 5). Oxaloacetate M+1 is generated by incorporating the ¹³CO₂ with nonlabelled pyruvate (Fig. 5A). Oxaloacetate M+1 is then converted into PEP M+1 by introducing a phosphate group from ATP, catalyzed by PEPCK-C. PEP M+1 is then hydrolyzed by enolase to produce 2PG M+1, which is in equilibrium with 3PG M+1 in the presence of PGAM. 3PG M+1 is eventually converted into serine M+1. Indeed, consistent with the model that ¹³CO₂ recycling is substantial in vivo, 2PG/3PG M+1 was only observed in tumors after in vivo infusion, but not in cultured tumor cells (Fig. 5B). Subsequently, de novo serine biosynthesis from 3PG M+1 leads to the production of serine M+1 (Fig. 5B). These results suggest that ¹³CO₂ participates in the biosynthesis of serine in vivo.

Citrate M+1 is a potent marker of pyruvate carboxylase in vivo

Pyruvate anaplerosis is the counterpart to glutamine anaplerosis and allows the TCA cycle to continuously oxidize acetyl-CoA simultaneously to provide carbon backbones for biomass production. In the [U-¹³C]-glucose tracing experiment, [¹³C₃]-pyruvate (M+3) is produced through glycolysis. Pyruvate M+3 enters the TCA cycle through pyruvate dehydrogenase or pyruvate carboxylase, leading to the production of M+2 acetyl-CoA or M+3 oxaloacetate, respectively, which subsequently contributes to the production of citrate M+2 and M+3, respectively. Hence, citrate M+3 is usually used as a marker of pyruvate carboxylase in many studies¹. Surprisingly, the time-course experiment (Fig.

6) demonstrated that citrate M+1 is more abundant than M+3 and also appears earlier than M+3 in the liver, skeletal muscle and plasma of mice receiving [U-¹³C]-glucose tracing. Non-labeled pyruvate and [U-¹³C]-glucose-derived ¹³CO₂ produce oxaloacetate M+1 via pyruvate carboxylase. Oxaloacetate M+1 is then converted into citrate M+1. Citrate M+1 mirrors that in tissues (liver and skeletal muscle), and thus plasma citrate M+1 may serve as a useful biomarker of pyruvate carboxylase.

When taken together, these results suggest that even though ¹³CO₂ produced from the decarboxylation of ¹³C tracers is negligible in vitro, endogenous ¹³CO₂ produced in vivo is substantial and subsequently fixed into the TCA cycle, purine, and serine, resulting in M+1 isotopologues in different metabolic pathways, which are summarized in Fig. 7.

Discussion

Compared to metabolomics which detects changes in metabolite levels, stable isotope tracing is more informative, because it provides activity readout of metabolic enzymes in intact cells or whole organisms. Precisely relating metabolite enrichment patterns to a specific metabolic enzyme or metabolic pathway requires in-depth knowledge of metabolic reactions and biological systems in which the isotope tracing assay is performed. We performed [U-¹³C]-glutamine and [U-¹³C]-glucose tracing in sarcoma cells cultured in standard in vitro cell culture conditions and also in vivo in mice. We observed distinct enrichment patterns of TCA intermediates, featured by high-abundant M+1 species in mice, but not in cultured cells. Interestingly, observing high-abundant M+1 species in vivo recapitulates previously published results by other groups^{8, 11, 13-14}, but the reason for the difference in the M+1 species between in vivo and in vitro

experiments has not previously been explained. Our results are consistent with a model where the difference in M+1 species is due to the different sources of CO₂ in vivo and in vitro. Endogenous CO₂ in cell culture is diluted by exogenous CO₂ and NaHCO₃ from the artificial conditions within an incubator, and hence the ¹³C enrichment by [U-¹³C]-glutamine or [U-¹³C]-glucose is negligible. In contrast, CO₂ from endogenous metabolism is substantial in mice and our results indicate that CO₂ is labeled in the presence of [U-¹³C]-glucose or [U-¹³C]-glutamine. ¹³CO₂ re-enters the TCA cycle via pyruvate carboxylase-mediated pyruvate carboxylation, resulting in M+1-labeled TCA intermediates. High-abundant M+1 TCA species in multiple tissues of mice receiving [U-¹³C]-glucose were also observed by other groups. [U-¹³C]-glutamine may contribute to ¹³CO₂ production via α-ketoglutarate dehydrogenase- and isocitrate dehydrogenase-mediated decarboxylation reactions. [U-¹³C]-glucose may contribute to ¹³CO₂ production via oxidative pentose phosphate pathway or pyruvate dehydrogenase-mediated pyruvate decarboxylation. These findings suggest that special attention needs to be paid when M+1 species are used to track specific pathways. For example, [1-¹³C]-glutamine can be used as a tracer in cancer cells where citrate M+1 is used to monitor reductive glutaminolysis activity. This approach is acceptable when it is used in vitro because the endogenous CO₂ contribution is negligible in the presence of an exogenous CO₂ supplement. However, our findings reveal that this approach should be used with caution to estimate reductive glutaminolysis in vivo, because [1-¹³C]-glutamine will label CO₂, and citrate M+1 may come from pyruvate carboxylation and/or reductive glutaminolysis. In addition, adenosine M+1 may be misinterpreted as the contribution via one-carbon metabolism in vivo, but [U-¹³C]-glutamine is not likely

contributing to one-carbon metabolism, so adenosine M+1 is also due to recycling $^{13}\text{CO}_2$.

Replenishing the TCA cycle (a process termed anaplerosis) is important to maintain homeostasis. The carbon source of anaplerosis varies from tissue to tissue and is also dependent on physiological conditions (e.g., fast vs fed state). Pyruvate carboxylase has been identified to play an important role in regulating anaplerosis ¹¹. Hence, pyruvate carboxylase is involved in various types of diseases and is an emerging therapeutic target. Pyruvate carboxylase deficiency is a genetic disease present at birth, leading to damage to tissues and organs. Pyruvate carboxylase is also involved in tumorigenesis ^{10, 15-17}. High pyruvate carboxylase was shown to be correlated with glycemia ¹⁸. Tissue-specific inhibition of pyruvate carboxylase reduced plasma glucose concentrations and could be a potential therapeutic approach for nonalcoholic fatty liver disease, hepatic insulin resistance, and type 2 diabetes ¹⁸. Citrate M+3 is often used to reflect pyruvate carboxylase activity in [U- ^{13}C]-glucose tracing experiments, but our results show that citrate M+1 is more abundant than citrate M+3 in both tissues and plasma. Hence, citrate M+1 in plasma may be a better readout of tissue pyruvate carboxylase and may be useful to monitor the efficacy of treatments targeting pyruvate carboxylase.

Furthermore, CO_2 recycling may also affect the interpretation of the respiratory quotient (RQ), which is the ratio of CO_2 production to O_2 consumption. For every mole of glucose undergoing complete oxidation, 6 moles of CO_2 are produced and 6 moles of O_2 are consumed, so the RQ is 1. Each mole of palmitate produces 16 moles of CO_2 and consumes 23 moles of O_2 , so the RQ is close to 0.7 when palmitate is completely

oxidized. Hence, the RQ is used as an indicator of energy fuel (e.g. carbohydrate or fat) being metabolized in the body and helps to plan nutritional therapy. The RQ is determined by comparing exhaled gases to room air, but this approach does not take in vivo CO₂ recycling into consideration. Therefore, the RQ will be underestimated when CO₂ recycling is substantial, resulting in an inaccurate interpretation of fuel source utilization.

Conclusion

In summary, understanding enrichment patterns and individual isotopologue provides rich information on metabolic reactions. The production and recycling of ¹³CO₂ from the decarboxylation of [U-¹³C]-glucose or [U-¹³C]-glutamine is negligible in vitro due to dilution by the exogenous HCO₃⁻/CO₂ source, but in vivo the incorporation of endogenous ¹³CO₂ into M+1 TCA intermediates and other metabolites is substantial and should be considered. M+1 TCA intermediates in vivo also suggest the isotopic exchange of oxaloacetate, malate, fumarate, and succinate, indicating the reversibility of malate dehydrogenase, fumarase, and succinate dehydrogenase. These findings not only provide an interpretation of distinct labeling patterns of TCA intermediates in vivo and in vitro but also provide insights into the proper design of ¹³C tracing experiments and modeling when ¹³C tracers are used to study in vivo metabolism. In addition, using M+1 citrate in plasma as a readout of tissue pyruvate carboxylase potentially offers a noninvasive approach for the diagnosis of diseases of pyruvate carboxylase deficiency or monitoring of treatment efficacy when targeting pyruvate carboxylase is employed as a therapeutic strategy for the treatment of diseases.

Experimental procedures

Reagents

Optima LC-MS grade of ammonium acetate, water, acetonitrile and methanol were purchased from Fisher Scientific. [U-¹³C₆]- glucose and [U-¹³C₆]- glutamine were obtained from Cambridge Isotope Laboratories. RPMI 1640 medium and Fetal Bovine Serum (FBS) were obtained from Thermo Fisher Scientific. Dialyzed FBS was obtained from Thermo Fisher Scientific. Jugular vein catheters, vascular access buttons, and infusion equipment were purchased from Instech Laboratories.

Cell culture

Mouse primary sarcoma cell lines were generated from Pax7CreER-T2, p53^{FL/FL}, LSL-Nras^{G12D} tumors as previously described ¹⁹. Mouse sarcoma-derived cell lines were cultured in a 10 cm dish with full growth medium containing RPMI 1640 supplemented with 10 % FBS. The cell incubator was set at 37 °C supplemented with 5% CO₂. Cells were then seeded into 6 well plates. After overnight incubation in the full growth medium, the old medium was replaced with 1.5 ml of RPMI 1640 (supplemented with 10 % dialyzed FBS) containing 11.1 mM [U-¹³C]-glucose or 2 mM [U-¹³C]-glutamine. After 3 hours of ¹³C tracing, intracellular metabolites were harvested.

Animal Models

All animal procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at Duke University. The mouse model of soft-tissue sarcoma was generated on a mixed background (129/SvJae and C57BL/6) using a combination of

alleles that were previously described: Pax7^{CreER-T2} ²⁰, p53^{FL/FL} ²¹, LSL-Nras^{G12D} ²² and ROSA26^{mTmG}. Primary mouse soft tissue sarcomas were generated in the mouse hind limb as previously described ¹⁹ by intramuscular (IM) injection of (Z)-4-hydroxytamoxifen (4-OHT). 4-OHT was dissolved in 100% DMSO at a concentration of 10 mg/ml and 50 µl of the solution was injected into the gastrocnemius muscle.

In vivo ¹³C glucose and glutamine infusions

To perform in vivo nutrient infusions, chronic indwelling catheters were placed into the right jugular veins of mice, and animals were allowed to recover for 3-4 days prior to infusions. Mice were infused with [U-¹³C]-glucose for 3 hours at a rate of 20 mg/kg/min (150 µl/hour). Blood was collected via the tail vein at 0, 30 min, 1, 1.5, 2, 2.5, and 3 hours. The plasma was collected by centrifuging blood at 3,000g for 15 min at 4 °C. At the end of infusions, tissues were snap-frozen in liquid nitrogen and stored at -80 °C for further analyses. [U-¹³C]-glutamine (Cambridge Isotope Laboratories) was infused for 3 hours at a rate of 6 mg/kg/min (200 µl/hour).

HPLC method. The analysis of metabolites in mouse tissues and plasma was performed using Ultimate 3000 UHPLC (Dionex), while the analysis of metabolites from cultured cells was performed using Vanquish UHPLC (Thermo Fisher Scientific). A hydrophilic interaction chromatography method (HILIC) with an Xbridge amide column (100 x 2.1 mm i.d., 3.5 µm; Waters) was used for compound separation at 25 °C. Mobile phase A: water with 5 mM ammonium acetate (pH 6.8), and mobile phase B: 100 % acetonitrile. Linear gradient is: 0 min, 85% B; 1.5 min, 85% B; 5.5 min, 35% B; 6.9 min,

35% B; 10.5 min, 35% B; 10.6 min, 10% B; 12.5 min, 10% B; 13.5 min, 85% B; 17.9 min, 85% B; 18 min, 85% B; 20 min, 85% B. Due to the instrumentation difference between Ultimate 3000 UHPLC and Vanquish UHPLC, different flow rates were used. For Ultimate 3000 UHPLC, the flow rate is: 0-5.5 min, 0.15 ml/min; 6.9-10.5 min, 0.17 ml/min; 10.6-17.9 min, 0.3 ml/min; 18-20 min, 0.15 ml/min. For Vanquish UHPLC, the flow rate is: 0-5.5 min, 0.11 ml/min; 6.9-10.5 min, 0.13 ml/min; 10.6-17.9 min, 0.25 ml/min; 18-20 min, 0.11 ml/min.

Mass Spectrometry. The analysis of metabolites in mouse tissues and plasma was performed using Q Exactive Plus mass spectrometer (Thermo Fisher Scientific), while the analysis of metabolites from cultured cells was performed using Orbitrap Exploris 480 mass spectrometer (Thermo Fisher Scientific). Both mass spectrometers are equipped with a HESI probe and operated in the positive/negative switching mode. When Q Exactive Plus mass spectrometer was used, the relevant parameters are as listed: heater temperature, 120 °C; sheath gas, 30; auxiliary gas, 10; sweep gas, 3; spray voltage, 3.6 kV for positive mode and 2.5 kV for negative mode; capillary temperature, 320°C; S-lens, 55. The resolution was set at 70,000 (at m/z 200). Maximum injection time (max IT) was set at 200 ms and automated gain control (AGC) was set at 3×10^6 . When Exploris 480 mass spectrometer was used, the relevant parameters are as listed: vaporizer temperature, 350 °C; ion transfer tube temperature, 300 °C; sheath gas, 35; auxiliary gas, 7; sweep gas, 1; spray voltage, 3.5 kV for positive mode and 2.5 kV for negative mode; RF-lens (%), 30. The resolution was set at 60,000

(at m/z 200). Automatic maximum injection time (max IT) and automated gain control (AGC) were used.

Metabolite extraction from cultured cells, tissues and plasma. To harvest intracellular metabolites, cells were briefly washed with ice cold saline (0.9% NaCl, 1ml, twice) and immediately placed on dry ice before they were extracted into 1 ml extraction solution composed of 80% methanol/water (pre-cooled in -80 °C freezer). Samples were centrifuged at 20,000 g for 10 min at 4 °C, and the supernatant was split into two Eppendorf tubes before drying in a speed vacuum concentrator (Labcono). The dry pellets were reconstituted into 30 μ l sample solvent (water: methanol: acetonitrile, 2:1:1, v/v) and 3 μ l was injected into the LC-HRMS. The tumor sample was first homogenized in liquid nitrogen and then 5 to 10 mg was weighed in a new Eppendorf tube. Ice cold extraction solvent (250 μ l) was added to the tissue sample, and a pellet mixer was used to further break down the tissue chunk and form an even suspension, followed by the addition of 250 μ l to rinse the pellet mixer. After incubation on ice for an additional 10 min, the tissue extract was centrifuged with a speed of 20,000 g at 4 °C for 10 min. The dry pellets were reconstituted into 30 μ l (per 3 mg tissue) sample solvent (water:methanol: acetonitrile, 2:1:1, v/v), and 3 μ l was injected to LC-HRMS. 5 μ l mouse plasma was mixed with 5 μ l water, and 40 μ l ice cold methanol was added. After vortex for 1 min, the mixture was centrifuged with a speed of 20,000 g at 4 °C for 10 min, and 3 μ l was injected to LC-HRMS.

Data analysis and statistics

LC-MS peak extraction and integration were performed using commercially available software Sieve 2.0 (Thermo Fisher Scientific). The integrated peak area was used to calculate ^{13}C enrichment. Natural abundance correction was performed using software R with Bioconductor R package IsocorrectionR²³. All data are represented as mean $\pm$ SD. All p values are obtained from the student's t-test two-tailed using GraphPad Prism 8 unless otherwise noted.

Acknowledgments

We thank Clay Rouse, DVM for consultation with the jugular vein surgical procedure. Mice were generously provided by Anton Berns (p53^{FL}) and Chen-Ming Fan (Pax7^{CreER-T2}). We thank North Carolina State University (NCSU) for financial support. We thank METRIC staff at NCSU for instrument maintenance. This work was supported by the National Institutes of Health R35CA197616 (D.G.K.) T32CA093240 (D.E.C.), Duke Cancer Institute Pilot Grant P30 CA014236 (D.G.K), a grant from the Slifka Foundation and Wendy Walk Foundation (D.G.K).

Author contributions

D.E.C. performed all animal experiments. X.L. and L.D. performed *in vitro* experiments and LC-MS analysis. X.L., L.D., D.E.C., J.W.L, and D.G.K. participated in experimental design. X.L, L.D., and G.S. interpreted results and wrote the manuscript. All authors provided input on the manuscript.

Conflict of interest

DGK is a cofounder of XRAD Therapeutics, which is developing radiosensitizers. DGK is on the scientific advisory board of Lumicell, which is developing intraoperative imaging technology. DGK is a recipient of a Stand Up To Cancer (SU2C) Merck Catalyst Grant studying pembrolizumab and radiation therapy in sarcoma patients. DGK has received research funding and/or reagents from XRAD Therapeutics, Merck, Amgen, Bristol Myers Squibb, Varian Medical Systems, and Calithera. These interests are outside the scope of the current manuscript. Other authors declare that they have no conflicts of interest with the contents of this article.

References

1. Jang, C.; Chen, L.; Rabinowitz, J. D., Metabolomics and Isotope Tracing. *Cell* **2018**, *173* (4), 822-837.
2. Wilkinson, D. J., Historical and contemporary stable isotope tracer approaches to studying mammalian protein metabolism. *Mass Spectrom Rev* **2018**, *37* (1), 57-80.
3. Buescher, J. M.; Antoniewicz, M. R.; Boros, L. G.; Burgess, S. C.; Brunengraber, H.; Clish, C. B.; DeBerardinis, R. J.; Feron, O.; Frezza, C.; Ghesquiere, B.; Gottlieb, E.; Hiller, K.; Jones, R. G.; Kamphorst, J. J.; Kibbey, R. G.; Kimmelman, A. C.; Locasale, J. W.; Lunt, S. Y.; Maddocks, O. D.; Malloy, C.; Metallo, C. M.; Meuillet, E. J.; Munger, J.; Noh, K.; Rabinowitz, J. D.; Ralser, M.; Sauer, U.; Stephanopoulos, G.; St-Pierre, J.; Tennant, D. A.; Wittmann, C.; Vander Heiden, M. G.; Vazquez, A.; Voutsden, K.; Young, J. D.; Zamboni, N.; Fendt, S. M., A roadmap for interpreting (13)C metabolite labeling patterns from cells. *Curr Opin Biotechnol* **2015**, *34*, 189-201.
4. Yuan, M.; Kremer, D. M.; Huang, H.; Breitkopf, S. B.; Ben-Sahra, I.; Manning, B. D.; Lyssiotis, C. A.; Asara, J. M., Ex vivo and in vivo stable isotope labelling of central carbon metabolism and related pathways with analysis by LC-MS/MS. *Nat Protoc* **2019**, *14* (2), 313-330.
5. Metallo, C. M.; Gameiro, P. A.; Bell, E. L.; Mattaini, K. R.; Yang, J.; Hiller, K.; Jewell, C. M.; Johnson, Z. R.; Irvine, D. J.; Guarente, L.; Kelleher, J. K.; Vander Heiden, M. G.; Iliopoulos, O.; Stephanopoulos, G., Reductive glutamine metabolism by IDH1 mediates lipogenesis under hypoxia. *Nature* **2011**, *481* (7381), 380-4.
6. Mullen, A. R.; Wheaton, W. W.; Jin, E. S.; Chen, P. H.; Sullivan, L. B.; Cheng, T.; Yang, Y.; Linehan, W. M.; Chandel, N. S.; DeBerardinis, R. J., Reductive carboxylation supports growth in tumour cells with defective mitochondria. *Nature* **2011**, *481* (7381), 385-8.
7. Cairns, R. A.; Harris, I. S.; Mak, T. W., Regulation of cancer cell metabolism. *Nat Rev Cancer* **2011**, *11* (2), 85-95.
8. Sun, R. C.; Fan, T. W.; Deng, P.; Higashi, R. M.; Lane, A. N.; Le, A. T.; Scott, T. L.; Sun, Q.; Warmoes, M. O.; Yang, Y., Noninvasive liquid diet delivery of stable isotopes into mouse models for deep metabolic network tracing. *Nat Commun* **2017**, *8* (1), 1646.
9. Cheng, T.; Sudderth, J.; Yang, C.; Mullen, A. R.; Jin, E. S.; Mates, J. M.; DeBerardinis, R. J., Pyruvate carboxylase is required for glutamine-independent growth of tumor cells. *Proc Natl Acad Sci U S A* **2011**, *108* (21), 8674-9.
10. Sellers, K.; Fox, M. P.; Bousamra, M., 2nd; Slone, S. P.; Higashi, R. M.; Miller, D. M.; Wang, Y.; Yan, J.; Yuneva, M. O.; Deshpande, R.; Lane, A. N.; Fan, T. W., Pyruvate carboxylase is critical for non-small-cell lung cancer proliferation. *J Clin Invest* **2015**, *125* (2), 687-98.
11. Cappel, D. A.; Deja, S.; Duarte, J. A. G.; Kucejova, B.; Inigo, M.; Fletcher, J. A.; Fu, X.; Berglund, E. D.; Liu, T.; Elmquist, J. K.; Hammer, S.; Mishra, P.; Browning, J. D.; Burgess, S. C., Pyruvate-Carboxylase-Mediated Anaplerosis Promotes Antioxidant Capacity by Sustaining TCA Cycle and Redox Metabolism in Liver. *Cell Metab* **2019**, *29* (6), 1291-1305 e8.

12. Fan, T. W.; Lane, A. N.; Higashi, R. M.; Yan, J., Stable isotope resolved metabolomics of lung cancer in a SCID mouse model. *Metabolomics* **2011**, 7 (2), 257-269.
13. Hui, S.; Ghergurovich, J. M.; Morscher, R. J.; Jang, C.; Teng, X.; Lu, W.; Esparza, L. A.; Reya, T.; Le, Z.; Yanxiang Guo, J.; White, E.; Rabinowitz, J. D., Glucose feeds the TCA cycle via circulating lactate. *Nature* **2017**, 551 (7678), 115-118.
14. Hasenour, C. M.; Rahim, M.; Young, J. D., In Vivo Estimates of Liver Metabolic Flux Assessed by (13)C-Propionate and (13)C-Lactate Are Impacted by Tracer Recycling and Equilibrium Assumptions. *Cell Rep* **2020**, 32 (5), 107986.
15. Lin, Q.; He, Y.; Wang, X.; Zhang, Y.; Hu, M.; Guo, W.; He, Y.; Zhang, T.; Lai, L.; Sun, Z.; Yi, Z.; Liu, M.; Chen, Y., Targeting Pyruvate Carboxylase by a Small Molecule Suppresses Breast Cancer Progression. *Adv Sci (Weinh)* **2020**, 7 (9), 1903483.
16. Phannasil, P.; Thuwajit, C.; Warnnissorn, M.; Wallace, J. C.; MacDonald, M. J.; Jitrapakdee, S., Pyruvate Carboxylase Is Up-Regulated in Breast Cancer and Essential to Support Growth and Invasion of MDA-MB-231 Cells. *PLoS One* **2015**, 10 (6), e0129848.
17. Cardaci, S.; Zheng, L.; MacKay, G.; van den Broek, N. J.; MacKenzie, E. D.; Nixon, C.; Stevenson, D.; Tumanov, S.; Bulusu, V.; Kamphorst, J. J.; Vazquez, A.; Fleming, S.; Schiavi, F.; Kalna, G.; Blyth, K.; Strathdee, D.; Gottlieb, E., Pyruvate carboxylation enables growth of SDH-deficient cells by supporting aspartate biosynthesis. *Nat Cell Biol* **2015**, 17 (10), 1317-26.
18. Kumashiro, N.; Beddow, S. A.; Vatner, D. F.; Majumdar, S. K.; Cantley, J. L.; Guebre-Egziabher, F.; Fat, I.; Guigni, B.; Jurczak, M. J.; Birkenfeld, A. L.; Kahn, M.; Perler, B. K.; Puchowicz, M. A.; Mancham, V. P.; Bhanot, S.; Still, C. D.; Gerhard, G. S.; Petersen, K. F.; Cline, G. W.; Shulman, G. I.; Samuel, V. T., Targeting pyruvate carboxylase reduces gluconeogenesis and adiposity and improves insulin resistance. *Diabetes* **2013**, 62 (7), 2183-94.
19. Zhang, M.; Qiu, Q.; Li, Z.; Sachdeva, M.; Min, H.; Cardona, D. M.; DeLaney, T. F.; Han, T.; Ma, Y.; Luo, L.; Ilkayeva, O. R.; Lui, K.; Nichols, A. G.; Newgard, C. B.; Kastan, M. B.; Rathmell, J. C.; Dewhirst, M. W.; Kirsch, D. G., HIF-1 Alpha Regulates the Response of Primary Sarcomas to Radiation Therapy through a Cell Autonomous Mechanism. *Radiat Res* **2015**, 183 (6), 594-609.
20. Lepper, C.; Conway, S. J.; Fan, C. M., Adult satellite cells and embryonic muscle progenitors have distinct genetic requirements. *Nature* **2009**, 460 (7255), 627-31.
21. Marino, S.; Vooijs, M.; van Der Gulden, H.; Jonkers, J.; Berns, A., Induction of medulloblastomas in p53-null mutant mice by somatic inactivation of Rb in the external granular layer cells of the cerebellum. *Genes Dev* **2000**, 14 (8), 994-1004.
22. Haigis, K. M.; Kendall, K. R.; Wang, Y.; Cheung, A.; Haigis, M. C.; Glickman, J. N.; Niwa-Kawakita, M.; Sweet-Cordero, A.; Sebolt-Leopold, J.; Shannon, K. M.; Settleman, J.; Giovannini, M.; Jacks, T., Differential effects of oncogenic K-Ras and N-Ras on proliferation, differentiation and tumor progression in the colon. *Nat Genet* **2008**, 40 (5), 600-8.
23. Heinrich, P.; Kohler, C.; Ellmann, L.; Kuerner, P.; Spang, R.; Oefner, P. J.; Dettmer, K., Correcting for natural isotope abundance and tracer impurity in MS-, MS/MS- and high-resolution-multiple-tracer-data from stable isotope labeling experiments with IsoCorrectoR. *Sci Rep* **2018**, 8 (1), 17910.

Figures and figure legends

Figure 1: Distinct ^{13}C labeling of TCA intermediates with $[\text{U}-^{13}\text{C}]$ -glutamine is observed in in vitro cell cultures and in tumors in vivo. (A) Schematic of in vivo and in vitro tracing analysis. (B) ^{13}C labeling patterns of metabolites from sarcoma cells (in vitro) fed with $[\text{U}-^{13}\text{C}]$ -glutamine medium for 3 hours (hrs) and sarcoma tissue from sarcoma-bearing mice (in vivo) receiving $[\text{U}-^{13}\text{C}]$ -glutamine infusion for 3 hrs. Data are presented as mean $\pm$ S.D. $n=5$ for animals and $n=3$ for cultured cells.

Figure 2: Distinct ^{13}C labeling of TCA intermediates with $[\text{U-}^{13}\text{C}]$ -glucose is observed in in vitro cell cultures and in tumors in vivo. (A) Schematic of in vivo and in vitro tracing assays. (B) ^{13}C labeling patterns of metabolites from sarcoma cells (in vitro) fed with $[\text{U-}^{13}\text{C}]$ -glucose medium for 3 hours (hrs) and sarcoma tissue from sarcoma-bearing mice (in vivo) receiving $[\text{U-}^{13}\text{C}]$ -glucose infusion for 3 hrs. Data are presented as mean $\pm$ S.D. $n=5$ for animals and $n=3$ for cultured cells.

Figure 3: M+1-labeled TCA intermediates are observed in non-tumor tissues in vivo. (A) ^{13}C labeling patterns of metabolites in skeletal muscle and liver from sarcoma-bearing mice receiving $[\text{U-}^{13}\text{C}]$ -glucose infusion for 3 hours. (B) ^{13}C labeling patterns of metabolites in different types of organs (data from Hui et al., Nature 2017)¹³. Data are presented as mean $\pm$ S.D. n=5 animals per group.

Figure 4: ¹³CO₂ contributes to adenosine biosynthesis. (A) Schematic of adenosine biosynthesis from various carbon sources. (B) ¹³C labeling pattern of adenosine from sarcoma cells fed with [U-¹³C]-glutamine/[U-¹³C]-glucose in vitro for 3 hours and labeling patterns of adenosine in sarcomas from sarcoma-bearing mice receiving [U-¹³C]-glutamine/[U-¹³C]-glucose infusion in vivo for 3 hours. Data are presented as mean ± S.D. n=5 for animals and n=3 for cultured cells. (R5P, Ribose 5-phosphate; AIR, Aminoimidazole ribotide; CAIR, 5-Amino-1-(5-phospho-D-ribosyl)imidazole-4-carboxylate.

Figure 5: ^{13}C contributes to serine biosynthesis. (A) Schematic of ^{13}C incorporation into serine via the serine biosynthesis pathway. (B) ^{13}C labeling pattern of 2PG/3PG and serine from sarcoma cells fed with $[\text{U}-^{13}\text{C}]$ -glutamine in vitro for 3 hours and labeling patterns of 2PG/3PG and serine in sarcomas from sarcoma-bearing mice receiving $[\text{U}-^{13}\text{C}]$ -glutamine infusion in vivo for 3 hours. Data are presented as mean $\pm$ S.D. $n=5$ for animals and $n=3$ for cultured cells. (PEP, Phosphoenolpyruvate; PEPCK-C, phosphoenolpyruvate carboxykinase; 2PG, 2-Phosphoglyceric acid; 3PG, 3-Phosphoglyceric acid; PGM, 2,3-bisphosphoglycerate-dependent phosphoglycerate mutase).

Figure 6: Plasma citrate M+1 is a potent biomarker of pyruvate carboxylase activity. Time course of citrate M+1 and M+3 in liver (A), skeletal muscle (B), and plasma (C) of mice receiving [U-¹³C]-glucose infusion for 3 hours (hrs). Data in (C) are presented as mean ± S.D. n=5 animals per group for the plasma sample only.

Figure 7: Proposed route of CO₂ incorporation into different metabolomic pathways.